

Studies on Antigenic Competition

I. ANTIGENIC COMPETITION BETWEEN THE Fc AND Fab FRAGMENTS OF RABBIT IgG IN MICE

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Summary. In the antibody response to rabbit IgG in BALB/c mice, antigenic competition has been demonstrated between Fc and Fab'. Similar competition was found when a mixture of Fc and Fab' was used as antigen. In both cases the presence of Fc suppressed the antibody response to Fab'. Competition was demonstrable only in the primary antibody response. The effect on competition of varying the routes and the time course of administration of the antigens was investigated.

INTRODUCTION

Antigenic competition, in which an immune response to one antigen is inhibited as a result of the injection of a second antigen, has been shown to occur in several systems dependent on a primary immune response. The best documented examples are those involving the primary humoral antibody response (Adler, 1964; Brody, Siskind and Walker, 1967; Amkraut, Garvey and Campbell, 1966; Eidinger, Khan and Millar, 1968). However, delayed hypersensitivity (Ben-Efraim and Liacopoulos, 1967; Schwartz and Leskowitz, 1969), the termination of immunological unresponsiveness with cross-reacting antigens, and the induction of autoimmunity (Weigle and High, 1967) have also been shown to be subject to competition. The work of these authors has shown the enormous variation in the results which can be obtained, depending on the antigens used, the doses and the relative times of administration; and there seems to be no easy framework into which the observations can be fitted. In this and following papers, an attempt is made to investigate the relationship between competition and such aspects of the immune response as suppression by passive antibody and the induction of tolerance; and to accommodate the results within current immunological theory.

For the context of antigenic competition the terms 'dominant' and 'suppressed' are used to refer respectively to the antigen whose presence suppresses the response to a second antigen, and to this second antigen.

MATERIALS AND METHODS

Rabbit γ -globulin

Cohn fraction II was obtained from Koch-Light Ltd. IgG was further purified from this on DEAE-cellulose in 0.01 M phosphate, pH 8.0.

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F(ab')₂ and Fab' fragments

Rabbit IgG was digested with pepsin, as described by Taussig (1970). After precipitation with 19 per cent Na₂SO₄, F(ab')₂ was further cleared of contamination by IgG and Fc fragments by chromatography on Sephadex G100. Fab' was prepared by treatment of F(ab')₂ with 0.01 M 2-mercaptoethanol, followed by 0.02 M iodoacetamide, and was purified on Sephadex G100.

Fab and Fc fragments

Papain digestion of rabbit IgG was carried out according to the method of Porter (1959). Separation of the fragments was by chromatography on CM-cellulose 52, in acetate buffer, pH 5.5. The Fc obtained was shown to be contaminated by Fab (Fig. 1a). In order to remove this contamination, an insoluble absorbent was prepared from a sheep anti-rabbit Fab serum, by the method of Avrameas and Ternynck (1967). Five ml of hyperimmune sheep anti-rabbit Fab serum was insolubilized at pH 4.5–5.0 with 0.2 ml of

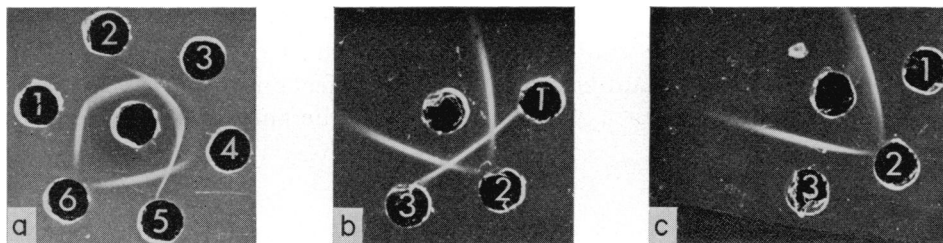


FIG. 1. (a) Double diffusion in agarose between goat anti-rabbit IgG (centre well) and IgG fragments. In outer wells: 1: papain Fab I; 2: papain Fab II; 3 and 4: Fc-containing fractions from CM-cellulose separation of papain digested IgG; 5: pepsin Fab'; 6: blank. The Fc-containing fractions are contaminated by Fab. (b) Double diffusion between goat anti-rabbit IgG, and in well 1: Fab I; 2: Fc-containing fraction after adsorption with insolubilized anti-Fab; 3: pepsin Fab'. Fc fraction is now free of Fab. (c) As Fig. 1b, but with sheep anti-rabbit Fab instead of goat anti-IgG. Fc-containing fraction again shows no precipitation.

ethyl chloroformate. When the reaction was complete (after about 1 hour) the precipitate was homogenized and washed repeatedly in 9 per cent saline to remove all traces of protein. The impure Fc preparation was absorbed with the insoluble anti-Fab. Fig. 1 (b and c) shows the product to be free of Fab.

Animals

BALB/c mice, bred at the Department of Pathology, Cambridge, were used throughout.

Immunization

Primary inoculation was generally with the antigen emulsified in Freund's complete adjuvant, intraperitoneally or subcutaneously. Secondary stimulation was with the antigen in saline.

Assay methods

Sera were assayed for anti-Fab' activity by the agglutination of Fab'-linked sheep erythrocytes. Monovalent anti-sheep red cell Fab', free of IgG and therefore without agglutinating titre, was prepared from hyperimmune sera by the methods described, and used to sensitize sheep red cells for agglutination by anti-Fab'.

Anti-Fc sera were assayed by agglutination of IgG-coated sheep erythrocytes in the presence of 0.1–0.5 mg/ml Fab' to inhibit agglutination by anti-Fab'. The agglutination could be inhibited by free Fc.

RESULTS

PRIMARY RESPONSE TO RABBIT IgG AND RABBIT F(ab')₂

BALB/c mice received 50 μ g IgG or 35 μ g F(ab')₂ emulsified in Freund's complete adjuvant, on day 0. The anti-Fab' and anti-Fc responses of the two groups are shown in Figs. 2 and 3. Mice that received rabbit IgG responded with anti-Fc production starting at about 8 days and reaching a plateau at about 14 days after inoculation (Fig. 2). The anti-Fab' response in three mice remained at a very low level for the first 18–20 days, and then increased steadily. This delay in the appearance of anti-Fab' antibodies was not observed in the group of animals which received F(ab')₂. A response began after 7 days

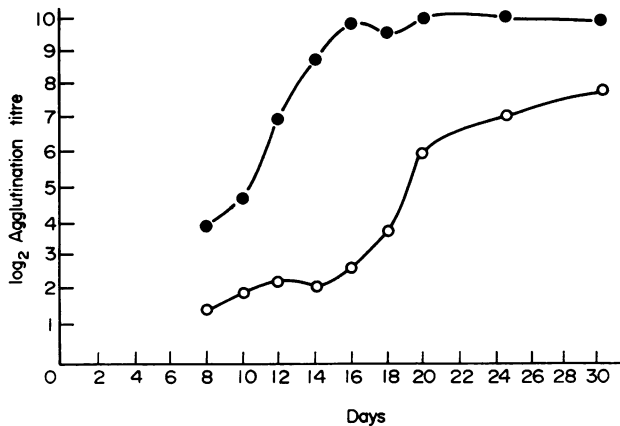


FIG. 2. Anti-Fc (●) and anti-Fab' titres (○), expressed as log₂ agglutination titres, of sera of BALB/c mice immunized on day 0 with rabbit IgG. Each point is mean of ten sera.

and reached a high titre by 12–14 days after immunization. Fig. 3 compares the anti-Fab' responses of the two groups. There was no increase in the anti-Fc or anti-Fab' response over a dose range of 50–500 μ g IgG per mouse.

These results suggested a state of antigenic competition in which Fc was dominant over Fab'. The inhibition tests summarized in Table 1 substantiate this. It was found that the agglutination of Fab'-coated sheep red cells produced by antisera raised to either Fab' or to IgG could be totally inhibited with either Fab' or with IgG. The Fab' determinants are thus the same on free Fab' as on IgG, and furthermore are available to antibody on the intact IgG molecule. While not proving that the Fab' determinants on IgG would also be available to cell-bound receptors, this at least argues that they are not buried within the IgG molecule.

Table 1 also shows that the agglutination of IgG-coated sheep red cells by anti-IgG sera taken 12 and 20 days after inoculation, could be 90 per cent inhibited by a mixture of purified Fab and Fc. This indicates that the early anti-IgG response is indeed directed against these antigens, rather than against a determinant dependent on the configuration of the whole IgG molecule.

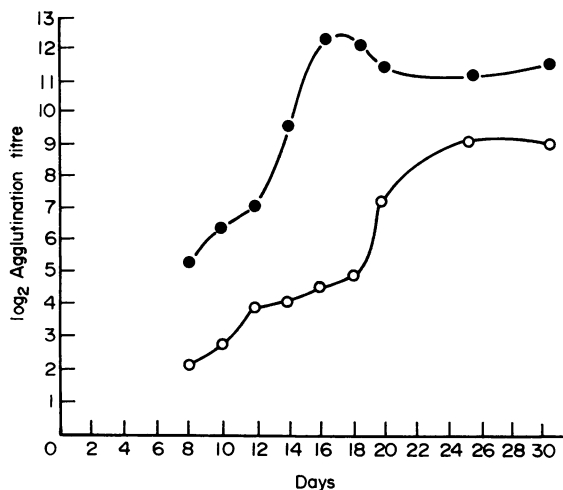


FIG. 3. Anti-Fab' titres, expressed as log₂ agglutination titres, of sera of BALB/c mice immunized on day 0 with rabbit IgG (○) or rabbit F(ab')₂ (●). Each point is mean of ten sera.

TABLE 1
ANTI-Fab' AND ANTI-IgG TITRES, EXPRESSED AS LOG₂ AGGLUTINATION TITRES IN THE PRESENCE OF FREE ANTIGENS AS INHIBITORS (CONCENTRATION 0.5 mg/ml)

Immunogen	Day	Inhibitor	Anti-Fab'	Anti-IgG
F(ab') ₂	10	—	3.8 (±0.87)	—
		F(ab') ₂	0	—
		IgG	0	—
	20	—	9.2 (±0.71)	—
		F(ab') ₂	0	—
		IgG	0	—
IgG	10	—	1.6 (±0.3)	4.2 (±0.5)
		F(ab') ₂	0	3.9 (±0.71)
		IgG	0	0
		Fab+Fc	0	0
	20	—	5.6 (±0.5)	9.5 (±1.0)
		F(ab') ₂	0	7.3 (±0.71)
		IgG	0	0
		Fab+Fc	0	2.5 (±0.5)
		—	0	0

Titres represent arithmetic means of 10, ± standard deviation.

RESPONSE TO MIXTURES OF IgG AND F(ab')₂, AND OF Fc AND F(ab')₂

BALB/c mice received mixtures of IgG and F(ab')₂, or of Fc and F(ab')₂ in different ratios (emulsified together in the same adjuvant). In the former case, the IgG component was varied from 50 to 500 μg, while the F(ab')₂ in the mixture varied from 33 to 100 μg (per mouse). At the molar ratio of 1 : 1, the mice received 50 μg IgG and 33 μg F(ab')₂ mixed together in Freund's complete adjuvant, and injected intraperitoneally. Table 2 shows the results. With a ratio of IgG to (F(ab')₂) of 3 : 1, there was a highly significant lowering of the anti-Fab' titre, showing that competition can occur without the antigens being linked together. A similar result was obtained with mixtures of Fc and F(ab')₂. A molar ratio of 3 : 1 (60 μg Fc mixed with 40 μg F(ab')₂) was again necessary to

TABLE 2
MEAN ANTI-Fab' TITRES OF GROUPS OF TEN BALB/c MICE IMMUNIZED ON DAY 0,
WITH RABBIT IgG AND F(ab')₂ MIXED IN DIFFERENT MOLAR RATIOS

Day	Molar ratio IgG:F(ab') ₂				
	10:1	5:1	3:1	1:1	1:3
12	1.3	2.3	1.6	6.7	7.1
17	2.8	2.1	2.9	9.7	10.2
22	6.9	8.6	7.2	11.2	10.8

Results as log₂ agglutination titres; arithmetic means.

TABLE 3
MEAN ANTI-Fab TITRES (LOG₂ AGGLUTINATION TITRES) OF GROUPS
OF TEN BALB/c MICE IMMUNIZED ON DAY 0 WITH MIXTURES OF
RABBIT Fc AND F(ab')₂ IN DIFFERENT MOLAR RATIOS

Day	Molar ratio Fc:F(ab') ₃			
	5:1	3:1	1:1	1:3
12	2.9	2.6	6.4	6.0
17	3.8	4.1	8.1	7.9
22	8.0	6.9	10.1	10.3

secure competition (Table 3). Fc is thus dominant over F(ab')₂ even when the antigens are separated. The anti-Fc titres were unaffected by the presence of the F(ab')₂ in the mixtures.

EFFECT OF ADMINISTERING COMPETING ANTIGENS IN SEPARATE SITES

Animals were inoculated with F(ab')₂ in Freund's complete adjuvant intraperitoneally, and at the same time with IgG in Freund's complete adjuvant subcutaneously over the

TABLE 4
MEAN ANTI-Fab' TITRES (AS LOG₂ AGGLUTINATION TITRES) OF GROUPS OF TEN
BALB/c MICE IMMUNIZED ON DAY 0 WITH RABBIT IgG AND F(ab')₂ IN SEPARATE
SITES AND IN DIFFERENT MOLAR RATIOS. IgG WAS GIVEN SUBCUTANEOUSLY ON
THE BACK AND F(ab')₂ WAS GIVEN INTRAPERITONEALLY, IN BOTH CASES IN
FREUND'S COMPLETE ADJUVANT

Day	Molar ratio IgG:F(ab') ₂		
	10:1	3:1	1:1
12	1.6 (±0.8)	1.9 (±0.7)	6.3 (±1.0)
17	2.6 (±1.1)	3.2 (±0.9)	7.6 (±1.2)
22	7.9 (±1.0)	7.3 (±1.1)	10.4 (±0.8)

Results as arithmetic means ± standard deviation.

scapulae. The ratios of the antigens were varied as in the previous experiment. As Table 4 shows, competition did occur under these circumstances, and showed exactly the same dose dependence as when the antigens were given mixed together, that is first appeared at a ratio of IgG to F(ab')₂ of 3 : 1. This point is noteworthy, because if competition were due to the ratio of concentrations of the antigens at any particular place, it would be expected

that a much higher ratio of IgG to F(ab')₂ would be required when the antigens were given in separate sites, than when mixed and given together at the same site.

EFFECT OF VARYING THE TIME OF ADMINISTRATION OF THE ANTIGENS

A further variable of antigenic competition is the relative time of injection of the two antigens. Tables 5 and 6 and Figures 4 and 5 show the results of an experiment in which rabbit IgG was injected at various times following the injection of rabbit F(ab')₂. In this case the antigens were emulsified in Freund's incomplete adjuvant, to avoid the complic-

TABLE 5

MEAN ANTI-Fab' TITRES (AS LOG₂ AGGLUTINATION TITRES) OF GROUPS OF TEN BALB/c MICE IMMUNIZED ON DAY 0 WITH RABBIT F(ab')₂ AND THEN GIVEN RABBIT IgG ON SUBSEQUENT DAYS. THE DOSES WERE 33 µg F(ab')₂ AND 150 µg IgG; BOTH ANTIGENS ADMINISTERED IN FREUND'S INCOMPLETE ADJUVANT

Days after F(ab') ₂ inoculation	IgG given on day					No IgG given
	2	4	5	6	8	
12	2.1 (0.8)	2.3 (0.5)	4.1 (1.2)	5.9 (0.9)	6.0 (0.9)	6.3 (0.8)
17	3.2 (0.8)	3.8 (1.0)	4.8 (0.9)	7.1 (1.3)	7.3 (0.6)	7.6 (0.9)
22	6.8 (1.2)	7.4 (1.0)	8.6 (0.7)	10.1 (1.0)	10.7 (1.1)	10.4 (1.2)

Results as arithmetic means, with standard deviations in parenthesis.

TABLE 6

MEAN ANTI-Fc TITRES (AS LOG₂ AGGLUTINATION TITRES) OF GROUPS OF TEN BALB/c MICE INJECTED WITH RABBIT IgG ON DIFFERENT DAYS AFTER INJECTION OF RABBIT F(ab')₂, WITH THE DAY OF INJECTION OF IgG CONSIDERED AS DAY 0. BOTH ANTIGENS WERE GIVEN IN FREUND'S INCOMPLETE ADJUVANT AND THE DOSES WERE 150 µg IgG AND 33 µg F(ab')₂

Days after IgG inoculation	F(ab') ₂ given on day				No F(ab') ₂ given
	-2	-4	-5	-6	
12	3.1 (0.6)	3.6 (0.7)	6.7 (0.9)	7.4 (1.1)	7.2 (0.9)
18	4.4 (0.9)	4.4 (0.9)	8.2 (1.1)	8.8 (1.3)	9.4 (1.0)
24	7.9 (1.0)	8.6 (1.2)	10.6 (0.9)	10.2 (1.3)	10.1 (0.75)

Results as arithmetic means, with standard deviations shown in parenthesis.

ating effects of immunization with the mycobacteria. Control groups, in which adjuvant alone was injected in place of one of the antigens, were included. The amount of each antigen injected was 33 µg for F(ab')₂ and 150 µg for IgG.

Table 5 and Fig. 4 show the effect on the anti-Fab' response of injecting the dominant antigen 2, 4, 5, 6 or 8 days after F(ab')₂. It will be noted that IgG could be given up to 5 days after F(ab')₂ and still result in antigenic competition. After this time, IgG had no effect on the anti-Fab' response. However, when the anti-Fc titres of these groups were examined, a considerable reduction in the antibody levels was noted in animals receiving IgG up to 4 days after F(ab')₂, as shown in Table 6 and Fig. 5. Thus, under these circumstances, antigenic competition results in the reduction of the response to both antigens. (Control groups which received adjuvant only, without the competing antigen,

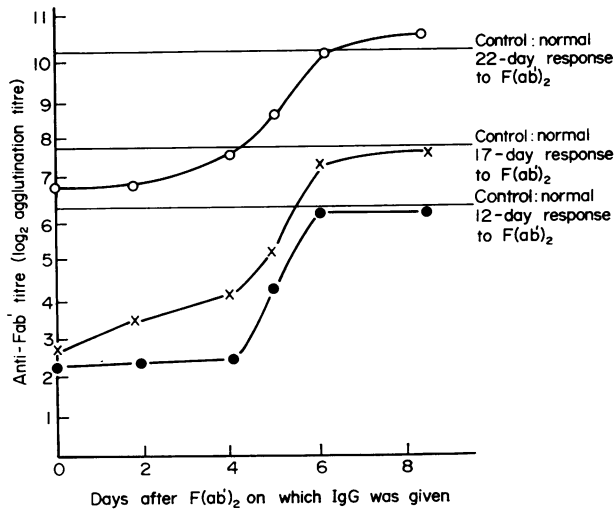


FIG. 4. Anti-Fab' titres in mice given rabbit IgG on different days after rabbit (F(ab')₂). Sera taken at 12 days (●), 17 days (×), and 22 days (○) after initial injection of F(ab')₂. Control lines show normal response levels to F(ab')₂.

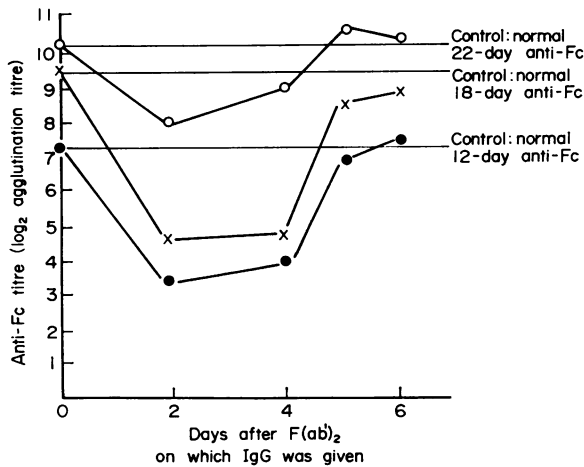


FIG. 5. Anti-Fc responses of BALB/c mice given rabbit IgG on different days after rabbit F(ab')₂. Sera taken 12 days (●), 18 days (×) and 22 days (○) after inoculation of IgG. Control lines show levels of anti-Fc in response to IgG in normal animals.

showed no reduction in titres.) The reduction in the anti-Fc titres may be the result of a non-specific effect noted by others (e.g. Radovich and Talmage, 1967) whereby an animal may show reduced responsiveness to any antigen injected a short time (2–10 days) after the injection of any other antigen.

SECONDARY RESPONSE TO RABBIT IgG AND RABBIT F(ab')₂

As noted in the introduction, antigenic competition has only been observed in systems dependent on a primary immune response. The following results confirm that the second-

ary response cannot be inhibited by antigenic competition. Fig. 6 shows the secondary response to IgG and $F(ab')_2$ measured as anti-Fab' titres in animals that had made a primary response to IgG or $F(ab')_2$ 5 weeks previously. It will be noted that the anti-Fab' titres are not affected by the mode of presentation of the Fab' antigen. However, animals primed with IgG showed a significantly lower secondary response to Fab' than animals primed with $F(ab')_2$ (Student's *t*-test, $P=0.99$). This is probably attributable to the smaller number of cells stimulated in the primary.

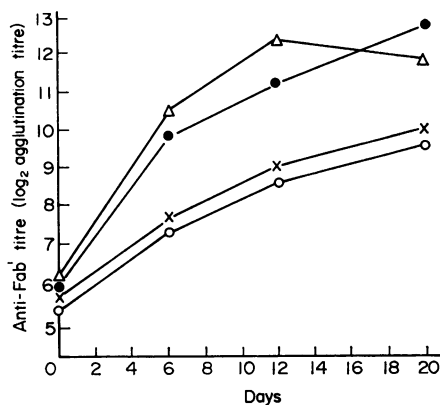


FIG. 6. Secondary response to rabbit IgG and $F(ab')_2$. Anti-Fab' titres of mice given a secondary immunization of either IgG or $F(ab')_2$ after a primary response to either IgG or $F(ab')_2$ 5 weeks previously. ○, primary immunization IgG, secondary IgG; ×, primary immunization IgG, secondary $F(ab')_2$; ●, primary immunization $F(ab')_2$, secondary IgG; Δ, primary immunization $F(ab')_2$, secondary $F(ab')_2$.

DISCUSSION

The experimental data described lead to the conclusion that the primary antibody response to rabbit Fab is suppressed by a simultaneous response to Fc. The effect was noted both when the antigens were linked together as rabbit IgG, or when administered as separate antigens mixed together. In this case, therefore, the term 'antigenic competition' is an appropriate one to describe the observations.

Studies of the response to heterologous γ -globulins in different species have often revealed differences in the magnitude of the response to the Fab and Fc antigens. Binaghi, Oriol and Boussac-Aron (1967), for instance, found that rabbits immunized with immunoglobulins from man, guinea-pig or rat, produced antibodies directed against both Fc and Fab fragments. Guinea-pigs and rats, on the other hand, responded mainly to the Fc antigens. In examining the causes for the lack of anti-Fab response, the authors state that rats immunized with $F(ab')_2$ alone also failed to respond, thus ruling out antigenic competition. In a similar study, Cerottini (1968) found that in hyperimmune sera of rats, guinea-pigs, goats and monkeys immunized with human γ -globulin, the antibody level to Fab was 2–4 times lower than to Fc, while in rabbits there was very little difference. The anti-Fab response in rats took 5 months to develop. In the present study, using rabbit IgG as antigen in BALB/c mice, there was no inability of the mice to respond to $F(ab')_2$ when administered as such in adjuvant. When given as IgG, however, the anti-Fab' response was both delayed by 7–10 days, and reached lower final titres.

In a recent study, Henney (1969) has shown that the early response to human γ -globulin in guinea-pigs is directed against neither Fab nor Fc, but is specific for a configurational determinant present only on the whole molecule. This possibility has been ruled out in the present investigation by the results of the inhibition tests, which showed that the agglutinating activity of early anti-IgG sera was completely inhibited by a mixture of purified Fab and Fc. Similarly, IgG could completely inhibit antisera raised to F(ab')₂, which tends to rule out the possibility that the suppression of the anti-Fab response with IgG as immunogen is due to the rather trivial reason that the determinants are buried within the IgG molecule and therefore simply not available to cell-bound receptors.

The observations with mixtures of IgG and F(ab')₂, and of Fc and F(ab')₂ provide further evidence that antigenic competition is occurring. Fc is clearly able to inhibit the response to Fab whether the antigens are linked together or not. These results also underline the dependence of antigenic competition on the relative doses of the competing antigens, as would be expected of a competitive effect. This should be borne in mind when drawing inferences from results where the doses of the antigens involved were not varied. Schechter (1968) for instance, reported that certain antigens would not compete with each other unless complexed to the same carrier molecules, yet did not attempt to vary the relative doses of the antigens used.

The observation that the competing antigens could be given in different sites and still affect the response to one another, is significant for the mechanism of competition. This result is in agreement with those of Adler (1964), and of Eidinger *et al.* (1968), but differs from the findings of Brody and Siskind (1969). In the latter results, the antigens had to be administered at the same site to compete successfully. In the present work, the significant point is that the antigens could be administered in widely separated sites (intraperitoneally and subcutaneously on the back) and still compete. If the mechanism of competition required both antigens to arrive simultaneously at the same site or cell one would not expect competition to occur under these circumstances, or, if distribution of the antigens throughout the body occurred, at least it would be predicted that the amount of the dominant antigen required would be much increased. Instead the results show that the lowest molar ratio at which competition could be demonstrated was the same, whether the antigens were administered together or in different sites. A probable implication of these observations is that the dominant antigen produces or removes a circulating factor which can affect the response to the suppressed antigen. In any case they tend to rule out an explanation in which interaction of antigens at a macrophage processing cell or similar was involved, as some authors have suggested (e.g. Brody *et al.*, 1967).

The order of administration of the antigens, as the results show, can affect the size of the responses obtained. The work of Radovich and Talmage (1967) and of Eidinger *et al.* (1968) has shown that antigens between which no competition occurs when they are administered together, can be made to compete with each other when one is given a suitable time before the other. In the work of these authors, the antigen given first became the dominant one, and inhibited the response to the antigen given after it. In the Fab/Fc system, two questions have been examined with this experimental design. One is whether a dominant antigen, such as Fc, can continue to exert its effect when given after the antigen which is usually suppressed, in this case Fab, and if so what is the latest time that a dominant antigen can be given and still suppress. The second is whether an antigen which is the suppressed one of a pair could itself become the dominant antigen simply by virtue of being given first.

The results given show that under these circumstances antigenic competition leads to the reduction of the response to both antigens, and is a combination of the continued dominance of the Fc given up to 5 days after $F(ab')_2$, and the expression of a new dominance of the Fab' itself given up to 4 days before the Fc. A possible objection which may be raised to this interpretation is that IgG has been used instead of free Fc, so that the presence of anti-Fab might affect the handling of IgG injected later. It seems very unlikely in the present case that sufficient anti-Fab is produced within the first 4 days after injection of $F(ab')_2$ to inhibit the response to the large amount of IgG given (150 μ g). The presence of anti-Fab in the serum at this time is barely detectable by agglutination or phage-linked assay. A further possibility, that IgG is exerting its effect simply by absorbing out anti-Fab, is also unlikely in view of the time-dependence of its action. Furthermore, experiments done on a smaller scale with purified Fc indicate that very similar results are obtained with the free fragment as with IgG.

A remarkable feature of these results is that IgG could be given 5 days after $F(ab')_2$ and still inhibit the anti-Fab response. It would be expected that the processes of cellular proliferation leading to antibody production to Fab' would by this time be well under way. Possibly this result reflects a continued requirement for antigenic stimulation throughout the inductive phases of the response, phases which could therefore be susceptible to antigenic competition.

Further experiments to examine the mechanism of competition will be described in a following paper.

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